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Antibiotic Penetration of and Bactericidal Activity within Endothelial Cells

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It has been observed that the number of cases of infective endocarditis arising in patients who have no previous identifiable cardiac abnormalities is increasing, suggesting that direct bacterial interactions with endothelium may occur. Furthermore, the prolonged natural history of endocarditis, need for lengthy therapy, and frequency of relapse suggest that intracellular bacteria that may be protected from antimicrobial action and host responses exist. Using high-performance liquid chromatography, we investigated the penetration of seven antibiotics used to treat *Staphylococcus aureus* infections into cultured human umbilical vein endothelial cells and the effect of these antibiotics on the intracellular killing of two strains of the organism. In general, endothelial cell penetration of lipophilic drugs, such as minocycline, ciprofloxacin, and rifampin, exceeded that of hydrophilic drugs, represented by nafcillin, cefazolin, cefuroxime, and vancomycin. Bacterial killing paralleled the intracellular penetration of all the antibiotics except rifampin, which concentrated well inside cells but had poor killing activity. However, when combined with the other antibiotics, rifampin potentiated their killing activity against intracellular *S. aureus*.

Infective endocarditis usually occurs in persons with preexisting lesions of cardiac valves. In those patients, initial bacterial colonization occurs on nonbacterial thrombotic vegetations, a meshwork of fibrin and platelets that develops on damaged endothelial surfaces (10, 38). However, it has been estimated that up to 30% of the infective endocarditis cases due to *Staphylococcus aureus* may occur in individuals who have no known preexisting heart disease (23, 42). Consequently, initiation of infective endocarditis in patients with healthy hearts probably involves direct interaction between bacteria and cardiac endothelial cells. Receptors that interact specifically with *S. aureus* have been identified on vascular endothelial cells, suggesting a mechanism whereby this normal endothelium may become colonized by infecting organisms (4, 21, 28, 43). We (14) and others (28, 32) have demonstrated that vascular endothelial cells cultured in vitro can phagocytose the adherent *S. aureus*. Although vascular endothelial cells are not considered "professional" phagocytes, electron microscopic studies have demonstrated the capacity of these cells to phagocytose nonbacterial elements in vivo (8). Moreover, as long ago as 1943, MacNeal demonstrated the presence of intracellular bacteria adjacent to cardiac vegetations in rabbits with experimental streptococcal endocarditis (30). It is, therefore, quite conceivable that staphylococci could be internalized by cardiovascular endothelium in vivo. If allowed to remain viable, as demonstrated by Vann and Proctor (45), or multiply intracellularly, as suggested by MacNeal (30), pathogens may eventually damage endothelial cells, thereby exposing the subendothelium and the thrombogenic underlying matrix and resulting in propagation of the lesion (13, 45). This may help explain the occasionally observed persistence and recurrence of *S. aureus* septicemia in the course of infective endocarditis (45) as well as the disseminated nature of endovascular infections in general (24). The interaction between host endothelial cells and pathogens is not limited to *S. aureus* and has been demonstrated for a wide variety of organisms, such as *Streptococcus* (6), *Salmonella* (37), *Rickettsia* (46), *Borrelia* (7), and *Candida* species (24, 35). Pathogens sequestered within endothelial cells, like other phagocytic tissue cells, may be protected from the action of antibiotics (36, 39, 44). Entry of an antibiotic into an endothelial cell is a prerequisite for its bactericidal activity against intracellular bacteria.

The present study was undertaken to do the following: (i) to compare the penetration of various antibiotics into human vascular endothelial cells and (ii) to examine the bactericidal activities of those antibiotics, alone and in combination, against intracellular *S. aureus*.

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MATERIALS AND METHODS

Endothelial cells. Cultured human umbilical vein endothelial cells were chosen as a model system because of the ready availability of these cells. This particular model has been used extensively in recent studies investigating the pathogenesis of infective endocarditis (4, 14, 21, 28, 32, 35, 43, 45). Previous studies have demonstrated that the in vitro adherence of *S. aureus* and other bacteria to umbilical vein-derived cells is equivalent to that with endothelial cells derived from cardiac valvular tissues (32). Segments of human umbilical cords were freshly obtained from a local hospital and transported to the laboratory in sterile phosphate-buffered saline (Sigma Chemical Co., St. Louis, Mo.). Umbilical veins were cannulated, flushed clear of blood, and filled with 0.028% collagenase (Boehringer Mannheim Corp., Indianapolis, Ind.) in Hanks balanced salt solution (HBSS; Sigma) for 30 min at room temperature (14, 20). Following this incubation, collagenase solutions containing cells were collected and centrifuged at 220 × *g* for 10 min. Cell pellets were resuspended in M199 cell

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TABLE 1. HPLC conditions for determining antibiotic concentrations

Antibiotic	Mobile phase	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)	Internal standard	Retention time for internal standard (min)
Nafcillin	30% ACN[a] + 70% 0.02M $NaOOCCH_3$ (pH 5.0)	1.5	224	8.0	5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	5.0
Cefazolin	10% ACN + 90% 0.05M KH_2PO_4 (pH 5.09)	1.0	254	11.0	Vancomycin	14.0
Cefuroxime	15% ACN + 85% 0.01M NH_3OOCCH_3 (pH 5.2)	1.0	254	6.8	Cefoperazone	14.8
Vancomycin	10% ACN + 90% 0.05M KH_2PO_4 (pH 5.09)	1.0	254	14.0	Cefazolin	11.0
Minocycline	45% ACN + 55% 0.05M KH_2PO_4 (pH 3.1)	1.0	353	3.0	Tetracycline	2.4
Ciprofloxacin	10% ACN + 90% 0.005M tetrabutyl ammonium hydrogen sulfate (pH 2.0)	1.0	280	14.0	Ofloxacin	10.6
Rifampin	40% ACN + 60% 0.05M KH_2PO_4 (pH 4.7)	1.0	340	6.2	Papaverine	9.5

[a] ACN, acetonitrile.

culture medium (Gibco Laboratories, Grand Island, N.Y.) that contained 20% heat-inactivated bovine calf serum (HyClone Laboratories, Logan, Utah) and endothelial cell growth factor (20 μg/ml; Biomedical Technologies Inc., Stoughton, Mass.). Cell suspensions were incubated in gelatin-coated (0.2% solution; Sigma) tissue culture plates (50 cm^2; Becton Dickinson Labware, Lincoln Park, N.J.) at 37°C in 5% CO_2 atmosphere. Endothelial cells had a characteristic cobblestone morphology (20) and usually became confluent in 10 to 14 days. All experiments were performed with cells that had been passaged only one or two times. For studies of the intracellular bactericidal activities of antibiotics, cells were passaged to 6-well tissue culture plates (9.6 cm^2 per well; Becton Dickinson).

Antibiotics. Experiments were performed with seven antibiotics of different classes that are used to treat infections due to *S. aureus*: nafcillin sodium, cefazolin sodium, cefuroxime sodium, vancomycin hydrochloride, minocycline hydrochloride, ciprofloxacin hydrochloride (Sigma), and rifampin sodium (Marion Merrell Dow Inc., Kansas City, Mo.). Minocycline was chosen for study because of some recently demonstrated in vitro (40) and in vivo (9, 26, 47) efficacy against *S. aureus* infections due to methicillin-resistant strains. The MBC of each antibiotic for the two strains of *S. aureus* was determined by standard macrodilution broth assays (31a).

Intracellular penetration of antibiotics. Endothelial cells cultured to confluence in tissue culture plates were washed with HBSS and then incubated in triplicate in bovine calf serum that contained one of the seven antibiotics at 37°C for 4 h. Pilot experiments demonstrated that the intracellular concentration of hydrophilic drugs within endothelial cells can be as low as 1% of the extracellular concentration; employing extracellular concentrations of hydrophilic drugs that are equivalent to clinically achievable serum levels resulted in some instances in which intracellular drug levels were either barely detectable or undetectable by high-performance liquid chromatography (HPLC) (data not shown; detection limit of these antibiotics ranged from 0.5 to 1 μg/ml). Incubating concentrations of antibiotics were, therefore, chosen with consideration for the degree of lipophilicity of the drug (which has previously been demonstrated to reflect the degree of intracellular penetration into other tissue cells [15, 16]) and the detection limit of intracellular drug concentration by HPLC: 300 μg/ml, for nafcillin, cefazolin, cefuroxime, and vancomycin; and 50 μg/ml, for minocycline, ciprofloxacin, and rifampin. Cells were washed with HBSS and detached with EDTA-trypsin (0.04% and 0.1%, respectively; Sigma). Cell suspensions were centrifuged and washed with HBSS three times, and then they were resuspended in 110 μl of HBSS. A 10-μl aliquot was used to determine the concentration of endothelial cells in suspension with a hemocytometer chamber. The average volume of endothelial cells was determined by incubating the cells with ^3H_2O and [^{14}C]polyethylene glycol (New England Nuclear Corp., Boston, Mass.), which distribute in the total water space and the extracellular compartment, respectively (25). Radioactivity was measured with a 1214 Rackbeta liquid scintillation counter (Pharmacia LKB Biotechnology Inc., Gaithersburg, Md.), and the difference reflected the intracellular volume. The ratio of the cell volume to the total volume of cell suspension was calculated by multiplying the concentration of cells in suspension by the average volume of an endothelial cell.

Extraction of antibiotics. One hundred-microliter aliquots of cell suspension were added to 100 μl of the internal standard (Table 1)–100 μl of HBSS. Cells were lysed by sonication for 30 min, and antibiotics were extracted with 800 μl of acetonitrile; preliminary experiments demonstrated no significant alteration of antibiotic concentrations during this prolonged sonication. After centrifugation at 13,000 × *g* for 5 min, the supernatant was decanted and dried under air, and the residue was redissolved in 100 μl of the appropriate mobile phase. Seventy-five-microliter samples were then injected into the HPLC system.

HPLC. Antibiotic levels were measured with a Waters liquid chromatograph (Millipore, Milford, Mass.) equipped with a model 700 satellite Waters intelligent sample processor, model 510 pump, μBondapack C_{18} column, model 486 UV absorbance detector, and model 820 Maxima control station. The separation conditions for each antibiotic are listed in Table 1.

A linear relationship was established between the antibiotic-to-internal standard peak height ratio and the concentration for each antibiotic in the appropriate ranges. The detection limits of antibiotics ranged from 0.1 to 1 μg/ml. After construction of standard curves, antibiotic controls were measured with a consistent accuracy of >90%. For each antibiotic, samples were analyzed the same day with an average intraday coefficient of variation of 7%. In pilot trials, aliquots of endothelial

cell suspensions that had not been incubated in antibiotics were added to the internal standard and a known amount of an antibiotic and then processed as described above; the recovery of antibiotics ranged from 80 to 94%.

Bacteria. In order to examine the possible differences in antibiotic-bacteria-endothelial cell interaction among strains of *S. aureus* that cause different clinical syndromes, the intracellular activities of antibiotics were studied by using two clinical isolates of *S. aureus*. The previously reported ENDO strain (33) was a blood isolate from a patient with documented endocarditis; the *S. aureus* SKIN strain was isolated from a patient with skin and soft tissue abscesses. Bacteria were grown overnight to logarithmic phase in Mueller-Hinton broth (MHB; Difco Laboratories, Detroit, Mich.) at 37°C. Organisms were harvested by centrifugation at 2,800 × *g* for 5 min, washed with HBSS, and then resuspended in MHB to the desired concentration immediately before use. Bacterial concentrations were estimated spectrophotometrically and confirmed by quantitative plate counts.

Intracellular activity of antibiotics. Confluent monolayers of endothelial cells grown in 6-well culture plates were washed with HBSS and then incubated with a bacterial suspension containing approximately 10^6 CFU/ml (bacteria/cell ratio of approximately 5:1) at 37°C for 3.5 h; this time was chosen because previous experiments using comparable bacterial concentrations demonstrated that endothelial cell-associated *S. aureus* strains reach an equilibrium by then (14). Wells were washed and treated with lysostaphin (1 μg/ml; Sigma) for 20 min at room temperature to kill bacteria adherent to cell surfaces; preliminary experiments demonstrated >99.99% killing of extracellular *S. aureus* exposed to lysostaphin under these conditions but no demonstrable effect of lysostaphin on ingested bacteria. The wells were again washed and incubated, in duplicate, with MHB containing twofold dilutions of one of the seven antibiotics; the lowest antibiotic concentration used was equal to the MBC for the specific strain of *S. aureus*. Control wells were also incubated with MHB that contained no antibiotics. After overnight incubation at 37°C, cells were washed and released by incubation with trypsin-EDTA for 10 min; preliminary studies revealed no effect of such concentrations of trypsin-EDTA on *S. aureus* and demonstrated an inhibitory effect of trypsin-EDTA on lysostaphin which may have adhered to cell surfaces. One-milliliter suspensions of endothelial cells from each well were disrupted by gentle sonication for 30 min (previously determined not to affect bacterial viability), serial dilutions were made with MHB, and 10-μl aliquots of the original suspensions and dilutions were plated onto Trypticase soy agar plates containing 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) at 37°C. Bacterial growth was quantitated the next morning and compared with that in control wells; the number of viable bacteria recovered from control wells ranged from 5×10^5 to 2×10^6 CFU/ml. The MBC for intracellular bacteria (IB) was defined as the incubating antibiotic concentration that resulted in ≥99.9% killing of organisms sequestered within endothelial cells.

RESULTS

Intracellular penetration of antibiotics. The ratios of intracellular concentrations to concentrations of antibiotics in serum ranged from 0.01 to 3.36 (Table 2). In general, the penetration of lipophilic drugs, such as minocycline, ciprofloxacin, and rifampin, into endothelial cells far exceeded that of hydrophilic compounds, including nafcillin, cefazolin, cefuroxime, and vancomycin (0.40 to 3.36 compared with 0.01 to 0.16, respectively).

TABLE 2. Penetration of antibiotics into endothelial cells[a]

Antibiotic	Incubating concentration (μg/ml)	Intracellular concentration (μg/ml)	Ratio[b]
Nafcillin	300	3.80 ± 0.64	0.01 ± 0.01[c]
Cefazolin	300	48.65 ± 14.21	0.16 ± 0.05
Cefuroxime	300	2.48 ± 0.94	0.01 ± 0.01[c]
Vancomycin	300	18.00 ± 17.09	0.06 ± 0.06
Minocycline	50	19.98 ± 0.59	0.40 ± 0.01
Ciprofloxacin	50	119.33 ± 10.02	2.39 ± 0.20
Rifampin	50	168.00 ± 1.73	3.36 ± 0.03

[a] Data are means from experiments performed in triplicate ± standard deviation.
[b] Intracellular to incubating concentration.
[c] Standard deviation was between 0.001 and 0.01.

Intracellular activity of antibiotics. Nafcillin, cefazolin, cefuroxime, and vancomycin required relatively higher concentrations in incubating serum to kill intracellular *S. aureus* of the ENDO strain than minocycline and ciprofloxacin did (Table 3); the ratios of the MBCs for IB to the conventionally determined MBCs ranged from 16 to 1,024 for the former drugs compared with 2 to 4 for the latter compounds. Experiments performed with the SKIN strain of *S. aureus* yielded similar results; hydrophilic drugs, in general, had lower intracellular bactericidal activities than lipophilic antibiotics. The only exception was rifampin, which concentrated very well inside endothelial cells (Table 2) but was less bactericidal for both strains of intracellular *S. aureus* (Table 3) when compared with the other lipophilic drugs, minocycline and ciprofloxacin.

Even though supraphysiologic concentrations of rifampin in the incubating medium were required to achieve ≥99.9% killing of intracellular *S. aureus* (128 μg/ml for the ENDO strain), the combination of rifampin at a concentration that simulates the usually achieved peak serum level (10 μg/ml) along with other antibiotics usually produced a beneficial effect. Table 4 demonstrates the observed reductions in the MBCs of nafcillin, vancomycin, and minocycline for IB when rifampin was combined with one of these three antibiotics. However, the combination of rifampin and ciprofloxacin was equivalent to ciprofloxacin alone in killing intracellular organisms.

TABLE 3. MBCs of single antibiotics for *S. aureus*[a]

Antibiotic	Bacterial strain	MBC (μg/ml)	MBC for IB (μg/ml)	Ratio[b]
Nafcillin	ENDO	2	64	32
	SKIN	0.25	8	32
Cefazolin	ENDO	1	64	64
	SKIN	4	32	8
Cefuroxime	ENDO	4	64	16
	SKIN	0.5	64	128
Vancomycin	ENDO	2	2,048	1,024
	SKIN	2	512	256
Minocycline	ENDO	8	32	4
	SKIN	4	16	4
Ciprofloxacin	ENDO	8	16	2
	SKIN	4	32	8
Rifampin	ENDO	0.5	64	128
	SKIN	1	64	64

[a] Data are means from experiments performed in duplicate.
[b] MBC for IB to MBC.

TABLE 4. Effect of combining rifampin with other antibiotics on an ENDO strain of *S. aureus*[a]

Antibiotic	MBC for IB (μg/ml)		Ratio[c]
	Antibiotic alone	Antibiotic + rifampin[b]	
Nafcillin	64	16	4
Vancomycin	2,048	128	16
Minocycline	32	8	4
Ciprofloxacin	16	16	1

[a] Data are means from experiments performed in duplicate.
[b] The rifampin concentration in incubating serum was 10 μg/ml. This corresponds to the average peak serum level usually obtained following an oral rifampin dose of 8 mg/kg.
[c] MBC of antibiotic alone to MBC of antibiotic and rifampin.

DISCUSSION

The concept that intracellular organisms may be protected from the host's immune response was first proposed by Rous and Jones in 1916 (36). Subsequently, this concept has become extended to antimicrobial therapy (18, 29, 41) and is now fairly well established in several different clinical situations, most notably infections due to *Mycobacterium tuberculosis* (29), *Legionella pneumophila* (19), and *S. aureus* (18). The prolonged natural course, need for extended antimicrobial therapy, and frequency of relapse suggest that such protected foci of bacteria may be present in the setting of infective endocarditis. Consequently, we have investigated the penetration of various classes of antibiotics used to treat *S. aureus* infections into endothelial cells. We have demonstrated that lipophilic drugs, including rifampin, ciprofloxacin, and minocycline, penetrate vascular endothelial cells to a greater extent than do hydrophilic drugs such as nafcillin, cefazolin, cefuroxime, and vancomycin.

The extent of penetration of these various drugs into vascular endothelial cells was in the same order as that previously shown for other cell types, including polymorphonuclear leukocytes, monocytes, alveolar macrophages (15, 16, 39), and fibroblasts (2). For instance, the intracellular-to-extracellular concentration ratio of rifampin for human phagocytes has previously been shown to range between 2 and 5 (16); we found a penetration ratio of 3.36. Ciprofloxacin has also been shown to concentrate two- to eightfold within phagocytes (44); our findings were comparable with a penetration ratio of 2.39. Conversely, beta-lactam antibiotics do not penetrate well into phagocytes (ratios of 0.07 to 0.9 for penicillin G [15, 39] and of <0.01 to 0.08 for cefazolin [15, 39]). We demonstrated similarly poor penetration into endothelial cells, with ratios of 0.01 and 0.16 for nafcillin and cefazolin, respectively.

The fact that a particular antibiotic concentrates within cells does not guarantee its activity in that setting. Clindamycin, for instance, is known to concentrate highly within cells by an energy-dependent process but has been reported to have significantly lower activity than what might be expected on the basis of the intracellular drug concentration (15, 16). We examined the ability of various antibiotics to kill intracellular organisms. Ciprofloxacin, for instance, achieved intracellular concentrations that exceeded extracellular levels; however, the MBC of this drug for IB was two to eight times higher than conventional MBCs. These results are in agreement with a previous report (12) that demonstrated the decreased bactericidal capacity of quinolones and other antimicrobial agents against slowly replicating and nonreplicating bacteria, especially *S. aureus*, as may be the case with bacteria residing within endothelial cells (45).

The MBC for IB usually paralleled the ability of the antibiotic to concentrate within endothelial cells, except for rifampin. The MBC of rifampin for IB was at least 64 times higher than the extracellular MBC. We have no explanation for this finding except to postulate that something within the endothelial cellular milieu may inactivate rifampin or affect the metabolic state of *S. aureus* such that it is less susceptible to the killing activity of rifampin. Alternatively, rifampin may be localized within a compartment separate from that containing *S. aureus*. These results with rifampin are in disagreement with those demonstrated with polymorphonuclear leukocytes, in which it has been postulated that the activity of rifampin is somehow enhanced by the intracellular physicochemical conditions prevailing at the site of infection (44). Despite the fact that rifampin exhibited poor intracellular killing when used as a single agent in this system, it lowered the MBCs of nafcillin, vancomycin, and minocycline for IB 4- to 16-fold when used in combination. Rifampin had no additional benefit when used in combination with ciprofloxacin, possibly because ciprofloxacin was concentrated so well within the cells.

Several factors that may influence the ability of various antibiotics to act on intracellular organisms have been suggested (15, 31, 39) and include the following. (i) The intracellular concentration of antibiotic that is achieved may differ from that which is necessary to kill the pathogen. According to our model, the intracellular levels of vancomycin that are expected with average concentrations in serum (0.06 × 20 μg/ml) would have been only about 50% of those necessary to kill either strain of *S. aureus*, for which the MBCs were 2 μg/ml. These data might help explain some of the concerns expressed regarding the efficacy of vancomycin used in the treatment of both experimental (5) and human infective endocarditis (27). (ii) The mechanism of action of the drug may not be compatible with the intracellular physicochemical milieu. The most notable example would be the aminoglycoside antibiotics, which concentrate largely within lysosomes, and consequently are less active within this relatively acidic compartment. (iii) The intracellular distributions of the antibiotic and pathogen may be different. For example, drugs such as the aminoglycosides may accumulate within the lysosome while the organism may be in the cytosol and not exposed to the antibiotic. In contrast, rifampin and ciprofloxacin are thought to enter phagocytes largely by passive diffusion, influenced by their relative lipophilicity, and probably distribute largely to the cytosol (44). (iv) The antibiotic may influence intrinsic cellular microbicidal capacity. (v) The process of bacterial phagocytosis may influence the uptake of the antibiotic.

In other situations, clinicians have made therapeutic choices based on the concept of microorganisms being sequestered within foci largely inaccessible to the host immune response or to antibiotics (1, 3, 31). Most notable for *S. aureus*, rifampin has been added to a beta-lactam or vancomycin in an attempt to eradicate *S. aureus* in settings as varied as arthritis (1), peritonitis (3), and chronic granulomatous disease (48). Combination antibiotic therapy including rifampin and a quinolone, both administered orally, has also been successfully used for treatment of right-sided staphylococcal endocarditis in intravenous drug users (11, 17).

Potential mechanisms of delivering higher concentrations of drugs into intracellular locations within phagocytes are being explored and may be useful for endothelial cells. Alteration of the chemical properties of penicillin G to produce more basic derivatives has been demonstrated to improve penetration, unfortunately, at the expense of antimicrobial activity (34). Encapsulation of antibiotics within liposomes, which then are internalized by phagocytic cells, is another promising ap-

proach; however, this mechanism is not likely to be of much benefit for vascular endothelial cells because of their low endocytic capacity and the inability of liposomes to traverse vascular endothelium (22).

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